The Impact of Genetic Markers on the Diagnosis of Lung Cancer: A Current Perspective

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Abstract: Lung cancer is the leading worldwide source of cancer-related death. It is acknowledged that prognosis and treatment outcomes in lung cancer might be improved by increasing the effectiveness of early-stage diagnosis. Several recently published studies have produced intriguing results regarding the detection of biomarkers in tumor samples, but also in easily accessible specimens such as sputum, plasma, and exhaled breath condensate. This review presents advances in genetic diagnostics of lung cancer, with particular reference to the clinical usefulness of individual biomarkers, specimens, and methods. The adequacy of their sensitivity and specificity for cancer screening and early detection is discussed in detail.

Key Words: Biomarkers, Bronchoalveolar lavage, Exhaled breath condensate, Lung cancer, Plasma DNA, Sputum.

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Lung cancer is currently regarded as one of the key epidemiological issues worldwide, with 1,200,000 new cases per year and only 10% (range, 5.5%–14.3%) of patients surviving their malignancy.[1,2] Although the prognosis in lung cancer is strongly correlated with disease stage at the time of diagnosis, no easily applicable methods exist for screening or early detection. It is hoped that molecular biology will eventually provide useful diagnostic biomarkers and tests, allowing reliable preselection of patients before, or in combination, with modern diagnostic imaging procedures such as fluorescence bronchoscopy, low-dose computed tomography (CT), and integrated positron emission tomography/CT. Molecular biology techniques might effectively estimate the expression of particular appointed genes not only in tumor cells, but also in other materials like sputum, bronchoalveolar lavage (BAL), and serum/plasma (Table 1). The efficiency of biomarker application to lung cancer diagnosis depends both on the validity of the selected marker (its sensitivity and specificity) and on the choice of accessible biological material.[3]

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TISSUE SPECIMENS FOR MOLECULAR TESTING

Biomarkers appropriate for a screening program should demonstrate a major difference between the tumor and normal tissue, correlate with cancer progression, and require no or a minimally invasive sampling technique with a low cost and high efficacy.[4] Several materials have been tested in the search for a useful and reliable source of biomarkers for screening and early diagnosis of lung cancer. Although some studies have produced promising data, no definite applications have yet been introduced into the clinical routine.

SPUTUM

It has been repeatedly demonstrated that cytologic evaluation of sputum samples due to its low sensitivity is an inadequate method for lung cancer screening and early diagnosis. Therefore, sputum examination serves as a good reminder of the advantages that biomarker analysis offers, especially in combination with other diagnostic methods.

Nuclear image analysis based on stoichiometric DNA-specific nuclear staining is currently perceived as one of the most promising novel biomolecular techniques. Implementation of the Feulgen-thionine reaction results in a linear relationship between the staining intensity and DNA quantity in a sample.[5] Palcic et al.[6] analyzed malignancy-associated changes in normal (nonmalignant) sputum cells by means of semiquantitative nuclear image analysis and demonstrated 45% sensitivity and 90% specificity in stage I lung cancer. In comparison, an evaluation performed using the standard Saccomano cytology method showed 14% sensitivity and 99% specificity. Developing an automated quantitative modification of this method has resulted in significantly improved sensitivity (75%–82%) and specificity (90%) of sputum cell evaluation.[7] Nuclear image analysis appears highly promising for improving or refining diagnosis beyond the use of a conventional sputum cytology examination.

Moreover, McWilliams et al.[8] reported that an automated quantitative sputum nuclear image analysis combined with spiral CT scanning in a routine lung cancer screening of a high-risk population of heavy smokers resulted in an additional benefit (detection rate of 2.1%) over high-resolution CT alone (detection rate of 1.8%). Furthermore, multiple screening, including autofluorescence bronchoscopy, further increased the overall detection rate to 3.1%. The results of that study also imply the potential positive economic and psychological effect of biomarker implementation. Preliminary molecular screening prior to the execution of invasive or

TABLE 1. Overview of the Key Biomolecular Techniques and the Most Promising Molecular Markers Analyzed within Various Tissue Specimens in Early Lung Cancer Detection Studies

Specimen		Biomolecular technique					
		Immunocytochemistry	**Cytogenetic Analysis**	**DNA/RNA Quantitation**	**Methylation Analysis**	**Microsatellite Analysis**	**Mutation Analysis**
	Assay	Immunostaining, immunofluorescence	Nuclear image analysis (Feulgen-thionine reaction), FISH	Spectrophotometry, colorimetry, fluorimetry, real-time qPCR, ELISA	MSP	PCR-based assays	PCR-SSCP, sequencing, hybridization
Sputum	Molecular marker	hnRNP overexpression	Nuclear morphology, DNA content, chromatin texture, aneusomy		p16, MGMT, RASSF1A, HOX A9, MAGE		*TP53*, K-*ras*
BAL		hnRNP overexpression		DNA, mRNA	p16, RARβ, FHIT, MGMT, CDH1	MSI, LOH	*TP53*, K-*ras*
EBC				Total DNA		MSI, LOH	*TP53*, K-*ras*
Blood				Total DNA/RNA, hTERT DNA, nRNP mRNA, nucleosomal DNA	p16, TMS1, RASSF1A, DAPK	MSI, LOH	*TP53*, K-*ras*

See references in text. BAL, bronchoalveolar lavage; CDH1, cadherin1 gene; DAPK, death-associated protein kinase; EBC, exhaled breath condensate; ELISA, enzyme-linked immunosorbent assay; FHIT, fragile histidine triad gene; FISH, fluorescence in situhybridization; hTERT, human telomerase catalytic component; hnRNP, heterogeneous nuclear ribonucleoprotein; LOH, loss of heterozygosity; MAGE, melanoma-associated antigen gene; MGMT, methylguanine methyl-transferase; MSI, microsatellite instability; MSP, methylation-specific polymerase chain reaction; PCR-SSCP, polymerase chain reaction–single-strand conformational polymorphism; qPCR, quantitative polymerase chain reaction; RAR, replication-associated repair gene; RASSF1A, RAS-associated domain family 1A; TMS1, target methylation inducing silencing.

radiographic diagnostic procedures should allow easier and less expensive positive identification of lung cancer patients. In the McWilliams et al.[8] study, none of the patients negatively verified by automated quantitative sputum nuclear image analysis were diagnosed with lung cancer.

Several other molecular techniques might prove useful for routine early lung cancer detection. Overexpression of heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 and B1 has shown promise for early detection in sputum. Tockman et al.[9] demonstrated that hnRNP A2/B1 immunostaining of archived preneoplastic sputum specimens detected preclinical lung cancer threefold more accurately than standard cytomorphology. Sputum hnRNP A2/B1 overexpression showed satisfactory sensitivity (82%) and specificity (65%), with a positive predictive value of 69% and negative predictive value of 20%. However, Sueoka et al.[10] noted that although 100% of stage I lung cancer tissue examined demonstrated positive staining with anti-hnRNP B1 antibody, only three of 12 sputum samples were positive. Apparently, although the reactivity of hnRNP B1 antibody was classified as strong in the sputum of lung cancer patients, its affinity might not be sufficiently high for large-scale application, and it is in need of further refinement.

The applicability of multitarget fluorescence in situ hybridization (FISH) to sputum analysis has also been recently assessed. FISH is the chromosomal instability evaluation technique that allows the simultaneous screening and assessment of changes in several distinct DNA sequences. Evaluation of the sputum specimens, obtained at least 12 months before lung cancer diagnosis, with the probe set for analysis of 5p15, 6p11-q11, 7p12, and 8q24, revealed aneusomy in 41% of the subjects (cutoff of two or more abnormal cells).[11] However, combining FISH with cytology improved the sensitivity to 83%,[12] with a concomitant decrease in the specificity from 94% to 80% (similar to cytology alone). However, Kettunen et al.[13] demonstrated that FISH sensitivity (50%) did not significantly exceed the sensitivity of sputum cytology (44%) in lung cancer diagnosis. Likewise, FISH analysis of 5p15, 6p11-q11, 7p12, and 8q24 sequences was not able to distinguish heavy tobacco smokers and asbestos-exposed workers from healthy nonsmokers (20%, 12%, and 27% positive individuals, respectively; cutoff of three or more abnormal cells). Thus, additional studies are needed to identify other hybridization targets (chromosomal loci) for FISH probes that would prove more useful for lung cancer risk assessment and early detection.

The methylation-specific polymerase chain reaction (MSP) is an extremely sensitive technique for assessing the methylation status of DNA promoter regions. Aberrant methylation of *p16* and other tumor-related genes has been repeatedly detected in sputum DNA and has been shown to be an early event in lung cancer.[14] Therefore, several biomarkers potentially useful for early diagnosis have been evaluated in sputum using the MSP method. Palmisano et al.[15] detected aberrant methylation of *p16* and/or *MGMT* promoters in sputum DNA of all 10 patients with squamous cell carcinoma (SCC) up to 3 years before clinical diagnosis, whereas only one patient showed positive sputum cytology. Concordance between *p16* and *MGMT* methylation in the primary SCC and paired sputum samples was 90% and 78%, respectively, for each gene. Furthermore, a comparative study on plasma and sputum samples from subjects grouped with respect to lung cancer risk revealed that methylation of three genes (*p16*, *MGMT*, and *RASSF1A*) was generally more common in sputum than in plasma. Similarly, the prevalence for *RASSF1A* and *MGMT* methylation was significantly greater in sputum than in plasma from lung cancer survivors (25% versus 7% and 36% versus 11%, respectively), whereas *p16* methylation occurred more frequently in sputum than in plasma from smokers (25% versus 13%).[16] Meanwhile, Circincione et al.[17] failed to identify early lung cancer patients with tumors detected by spiral CT using a panel of methylated *RARβ2*, *p16*, and *RASSF1A* markers in DNA from sputum. Methylation of both *p16* and *RASSF1A* genes was detected in the sputum of only one of 18 (5%) patients with paired tumor samples, and in one of 112 (0.9%) and 20 of 112 (17.8%) sputum samples, respectively, from cancer-free smokers. Moreover, *RARβ2* methylation was revealed in the sputum of CT-detected patients (8/18, 44.4%) and heavy smokers without cancer (58/112, 51.7%), suggesting that it is an exposure-related rather than tumor-specific marker. In contrast, a differently designed marker system revealed quite promising sensitivity in a methylation study on sputum samples from 22 patients with resected non-small cell lung cancer (NSCLC). Abnormal methylation in the cytologically negative sputum samples was displayed by 64% of *HOX A9*, 50% of *MAGE A1*, 41% of *MAGE B2*, and 27% of *p16* promoters.[18] Furthermore, 95.5% of the negative sputum samples from NSCLC patients showed abnormal methylation in at least one gene tested. However, the lack of a control group is a serious limitation for this study and prevents any prospective conclusions being drawn. Consequently, the diagnostic utility of the proposed marker set needs further confirmation. Note that several inaccuracies in the MSP procedure might generate false-positive results, and thus, a crossover study comparing different technical approaches for methylation detection is necessary before its further diagnostic implementation.

Although novel technologies create new challenging and promising perspectives, some very practical aspects should not be forgotten. The low yield of sputum cytology is a common problem, its induction is relatively time-consuming (15–30 minutes), and specific conditions for sample storage and transportation must be guaranteed. All these might affect the reproducibility of sputum analysis results and its usefulness for screening and early diagnostic purposes.

BAL

BAL is conducted by reaspirating the sterile saline solution infused into the airways of a patient undergoing bronchoscopy. Thus, BAL potentially provides samples containing a deeper epithelial component than that found in sputum samples.

As early as 1999, Ahrendt et al.[19] implemented PCR-based techniques to assess molecular markers in the BAL-derived cellular material from 50 patients with resectable

NSCLC. However, the frequency of most typical alterations in the *p53* and K-*ras* gene expression or *p16* promoter methylation was significantly lower in BAL than in tumor samples: 53 versus 100% for all examined markers, 39 versus 56% for the *p53* gene, 27 versus 33% for the K-*ras* gene, and 17 versus 63% for *p16* methylation. Similar results have been reported by Ferretti et al.[20] who applied a novel PCR-denaturant gradient gel electrophoresis method. Calculated concordance between tumor and paired BAL samples in that study was 46.6% and 43% for *TP53* and K-*ras*, respectively, demonstrating an overall low sensitivity. Recent reports concerning the use of real-time PCR for biomarker analysis in BAL samples have been slightly more reassuring.[21] Modified expression of the *APC*, *RASSF1A*, *MGMT*, and *GSTP1* genes, as well as the *CDH1* methylation observed in NSCLC tumor cells was demonstrated in parallel in approximately 68% of BAL material (at least one gene).

Note, however, that BAL has repeatedly been shown to have a significantly higher diagnostic value to that of sputum-derived material. Fielding et al.[22] confirmed a 96% sensitivity and 82% specificity of hnRNP A2/B1 overexpression in BAL specimens from patients with premalignant and malignant lung lesions. Importantly, hnRNP overexpression was shown in 41 of 80 specimens reported as negative in routine cytologic examinations. Similarly, Chan et al.[23] directly compared the sensitivity of MSP versus conventional cytology in detecting cancer cells in BAL samples. MSP biomarkers were positive in 35 of 41 patients with negative cytology and histologically confirmed malignant pulmonary lesions.

In summary, according to available data, molecular approaches to biomarker assessment in BAL-derived material are characterized by inadequate sensitivity, even if their specificity seems acceptable. This may be due to both an insufficient level of cellular tumor DNA in BAL and excessive contamination with normal DNA.[21] Indeed, cancer cells in BAL fluid are usually mixed with a large number of normal cells, such as alveolar macrophages, white blood cells, and normal epithelial cells.[20]

Therefore, the detection and analysis of DNA in cell-free lavage supernatant appear to be an interesting alternative. Carstensen et al.[24] found microsatellite alterations (MAs) in the intact cell-free DNA from BAL fluids in 47% of the lung cancer patients evaluated. The majority of DNA alterations found in the cell-free lavage supernatants was not detected in lavage cells, once again demonstrating the low accuracy of cytologic examination. Recently, extracellular mRNA isolated from cell-free lavage supernatant was also analyzed, both qualitatively and quantitatively, showing interesting results.[25]

Finally, the technical obstacles that limit the clinical applicability of BAL should be mentioned, with the requirement for bronchoscopy being the most important. In addition to its invasiveness, it cannot be repeated frequently, especially in high-risk patients. Additionally, genetic analysis of BAL might not be particularly helpful in detecting peripheral cancer. Thus, the more diagnostically convenient materials such as sputum, blood, and exhaled breath condensate (EBC) should be taken into account as first-line materials for the validation of relevant markers.

EBC

EBC is a reliable source of proteins, nucleotides, lipids, oxidants, and human DNA derived from the fluid lining the respiratory tract.[26] Genetic analysis has shown several *TP53* mutations (exons 5–8) in EBC from four of 11 NSCLC patients (36%), but not from healthy nonsmoking subjects.[27] Carpagnano et al.[28] analyzed EBC-derived DNA samples from 30 NSCLC patients and 20 healthy volunteers for the presence of MAs on chromosome locus 3p. The number of MAs (either microsatellite instability or loss of heterozygosity) in EBC DNA was significantly higher in NSCLC patients than in healthy controls with a comparable history of smoking. Interestingly, the most frequently altered 3p microsatellite in EBC DNA was D3S1300 located in the *FHIT* gene, thus supporting an association between *FHIT* inactivation and carcinogenesis.

Regardless of the above-mentioned findings, several advantages of EBC essential for its potential application in lung cancer diagnosis should be noted. EBC collection is an easy, repeatable, and totally noninvasive procedure, unrelated in any way to patient airway function. Likewise, the required volume of diagnostic material can be obtained in a very economical manner, without the need for a highly specialized hospital environment. However, standard procedures concerning sample collection and results presentation need to be established.[26]

Further research will show whether genomic and proteomic analysis of EBC has an impact on lung cancer detection. Nevertheless, it seems reasonable to pursue this approach and expand the biomarkers panel with (proto)oncogenes or suppressor genes, as well as protein factors.

PERIPHERAL BLOOD (SERUM/PLASMA)

Although BAL material examination remains disappointing and EBC applicability needs further evaluation, peripheral blood assessment as a reservoir of lung cancer biomarkers has proven surprisingly efficient.[29] From a technical point of view, blood seems an ideal candidate material for screening and early diagnostic programs. It is easily and cheaply accessible, and in district outpatient clinics outside large hospital centers, no need exists for additional personnel training to provide proper sampling. Therefore, extensive research projects are currently being conducted to evaluate in detail the diagnostic value of multiple biomarkers measured in the peripheral blood. Data from the most representative studies on serum/plasma genetic markers detected in the early lung cancer are summarized in Table 2.

Nanogram amounts of free DNA exist in the serum of healthy subjects, as well as in patients with chronic inflammatory and autoimmune diseases.[30] Nevertheless, free serum DNA concentration in lung cancer patients is usually several times higher, most likely due to the necrosis/apoptosis processes in the tumor tissue or circulating cancer cells.[29] In view of recently published studies, quantitative measurement of free DNA in serum/plasma might be considered a highly promising and very cost-effective biomarker for lung cancer screening and detection. Importantly, elevated amounts of

TABLE 2. Studies Evaluating Serum or Plasma Markers in Lung Cancer Patients and Controls

Reference	Tumor Type	Marker	s/p	Method	No. of Patients	Serum/Plasma Positive		No. of Controls	% Positive
						No.	%		
Fournie et al., 1995[52]	NSCLC/SCLC	Total DNA	p	Nick translation	68	36	53	26	0
Sozzi et al., 2001[31]	NSCLC	Total DNA	p	Colorimetry	84	45	54	43	0
Herrera et al., 2005[33]	NSCLC	β-Actin	p	RT qPCR	25	12	48	11	0
Sozzi et al., 2003[32]	NSCLC	hTERT	p	RT qPCR	100	69	69	100	2
Pelosi et al., 2006[51]	NSCLC	hTERT mRNA	p	RT qPCR	34	4	12	10	0
Kopreski et al., 2001[48]	NSCLC	5T4 mRNA	s	RT-PCR	14	6	43	25	12
Fleischhacker et al., 2001[49]	NSCLC/SCLC	Her2/neu mRNA	s	RT-PCR	18	7	39	12	0
		hnRNP B1 mRNA			18	14	78	12	0
Sueoka et al., 2005[50]	NSCLC/SCLC	hnRNP B1 mRNA	p	RT qPCR	44	20	45	25	12
Esteller et al., 1999[37]	NSCLC	Methylation p16, DAPK, GSTpi, O6MGMT	s	MSP	22	12	54	11	0
Bearzatto et al., 2002[40]	NSCLC	Methylation p16	p	F-MSP	35	12	34	15	0
An et al., 2002[41]	NSCLC	Methylation p16	p	Seminested MSP	105	77	73	0	
Liu et al., 2003[53]	NSCLC/SCLC	Methylation p16	p	Seminested MSP	50	36	72	0	
Usadel et al., 2002[54]	NSCLC	Methylation APC	s/p	RT qPCR	89	42	47	50	0
Ramirez et al., 2003[38]	NSCLC	Methylation TMS1, RASSF1, DAPK	s	MSP	50	36	72	0	
Fujiwara et al., 2005[39]	NSCLC/SCLC	Methylation MGMT, p16, RASSF1A, DAPK, RAR-β	s	MSP	91	45	49	100[a]	8
Sozzi et al., 1999[55]	NSCLC	MAs (2 markers)	p	Radio PCR	87	35	40	14	0
Khan et al., 2004[56]	NSCLS/SCLC	MAs (3 markers	p	Radio PCR	86	59	69	120[a]	42
Bruhn et al., 2000[57]	NSCLC	MAs (3 markers)	p	Radio PCR	27	9	33	10	0
	SCLC				16	5	31		
Cuda et al., 2000[58]	NSCLC	MAs (3 markers)	s	Silver PCR	17	7	41	31	0
	SCLC	MAs (3 markers)			11	7	64		
Chen et al., 1996[36]	SCLC	MAs (3 markers)	p	Radio PCR	21	15	71	0	
Gonzalez et al., 2000[43]	SCLC	MAs (3 markers)	p	F-PCR	16	9	56	0	
Sanchez-Cespedes et al., 1998[45]	NSCLC	As (4 markers)	s	Radio PCR	22	6	28	0	
Sozzi et al., 2001[31]	NSCLC	MAs (5 markers)	p	F-PCR	38	9	24	43	0
Bearzatto et al., 2002[40]	NSCLC	MAs (5 markers)	p	F-PCR	34	11	32	0	
Beau-Faller et al., 2003[44]	NSCLS/SCLC	MAs (12 markers)	p	F-PCR	20	17	85	20	0
Gonzalez et al., 2000[43]	SCLC	*TP53* mutation	p	PCR-SSCP	35	6	17	13	0
Andriani et al., 2004[46]	NSCLC	*TP53* mutation	p	Hybridization	26	19	73	0	
Bearzatto et al., 2002[40]	NSCLC	K-*r as* mutation	s	Enriched PCR	35	0	0	0	
Ramirez et al., 2003[38]	NSCLC	K-*ras* mutation	s	Enriched PCR	50	12	24	0	

APC, adenomatous polyposis coli; DAPK, death-associated protein kinase; F-MSP, fluorescent MSP; F-PCR, fluorescent polymerase chain reaction; GSTp, glutathione S-transferase; Her2/neu, human epidermal growth factor receptor 2; hnRNP B1, heterogeneous nuclear ribonucleoprotein B1; hTERT, human telomerase catalytic component; MAs, microsatellite alterations; MSP, methylation-specific polymerase chain reaction; NSCLC, non-small cell lung cancer; O6MGMT, O6-methylguanine methyltransferase; PCR-SSCP, polymerase chain reaction–single-strand conformational polymorphism; p, plasma; radio(silver) PCR, polymerase chain reaction with detection of amplification products by autoradiography (silver staining); RASSF1A, RAS-associated domain family 1A; RT-PCR, reverse-transcriptase polymerase chain reaction; RT qPCR, real-time quantitative polymerase chain reaction; s, serum; SCLC, small-cell lung cancer; TMS1, target methylation–inducing silencing.

[a] Control group consisted of patients with nonmalignant pulmonary diseases.

circulating free DNA have been observed as early as the initial stages of lung tumor development.[31]

Sozzi et al.[31] measured circulating plasma DNA using a simple colorimetric assay and reported that 45% of 84 NSCLC patients exhibited up to 17 times higher levels of plasma DNA than healthy controls (optimized cutoff, 250 ng/ml). It has been shown that quantification of plasma DNA with optimally selected cutoff might be a valuable tool to discriminate patients from healthy individuals. Consequently, the same research group has applied quantitative real-time PCR of the human telomerase reverse transcriptase (*hTERT*) gene to measure cancer-derived DNA and to discriminate lung cancer patients from healthy smoking and nonsmoking controls. Very satisfactory sensitivity (90%) and specificity

(86%) have been demonstrated with a positive predictive value of 90% and negative predictive value of 90%.[32] Sixty-nine of 100 evaluated NSCLC patients and two of 100 controls showed elevated circulating DNA levels, which were eight times higher in the plasma of patients than controls (24.3 versus 3.1 ng/ml). The calculated relative risk of lung cancer was 85 times higher in subjects with a high DNA concentration. The striking difference in circulating DNA plasma levels reported in both studies might also be attributable to the sensitivity of the method used because colorimetric assays detect all DNA fragments available in the sample, whereas quantitative PCR measures only amplifiable DNA (*hTERT*).[33]

Real-time PCR can be regarded as the standard method currently available for DNA quantification. It allows simultaneous amplification and detection of specific DNA sequences by monitoring the fluorescence of dyes or probes introduced into the reaction in proportion to the amount of product formed. Consequently, real-time PCR is characterized by high accuracy, reproducibility, and time-effectiveness. To validate the method, Gautschi et al.[34] evaluated the impact of several essential factors on the reproducibility of DNA measurement: blood collection tube brand, DNA concentration assessment technique, DNA quantification procedures, and interassay operations. Results have indicated that the median variation is relatively low (4%, 25%, 20%, and 24%, respectively), allowing comparable and repeatable results in independent centers.

Additionally, it has been suggested that plasma DNA is superior to serum-derived material in terms of its diagnostic value. Plasma, which is less contaminated with DNA of nontumor origin (serum may contain a variable fraction of DNA derived from in vivo and in vitro damaged hematopoietic cells), is considered a more reliable source for the analysis of tumor-specific genetic and epigenetic alterations in circulating DNA.[34]

Extrapolating data from ovarian cancer studies, it has been assumed that a diagnostic panel combining free DNA assessment, together with analysis of other gene(s) modification(s) typical for lung cancer, might prove extremely efficient.[35] Subsequently, several studies have clearly demonstrated that identification of various genetic alterations related to human lung cancer in the circulating serum/plasma DNA is feasible.[36,37] So-called tumor molecular signatures, markers characteristic for primary tumor, such as *MSI*, *TP53*, and K-*ras* mutations, tumor suppressor genes, and DNA repair gene hypermethylation, have been identified in circulating DNA, confirming their tumor origin.

The abnormal methylation of four oncogenes (*p16*, *DAPK*, *GSTpi*, *O6MGMT*) was first observed by Esteller et al.[37] in the serum DNA from 11 of 22 (50%) NSCLC patients using the MSP technique. Likewise, Ramirez et al.[38] confirmed a high frequency of *RASSF1A*, *DAPK*, and *TMS*-1 (34%, 45%, and 35%, respectively) methylated DNA, allowing identification in 72% of 50 NSCLC patients (at least one methylated gene). They also demonstrated a highly significant correlation between the methylation rate in tumor tissue and serum. Similarly, Fujiwara et al.,[39] who analyzed five markers (*MGMT*, *p16*, *RASSF1A*, *DAPK*, *RARβ*) in serum DNA from lung cancer and nonmalignant pulmonary patients, demonstrated that the methylation ratio of all examined genes was significantly higher in lung cancer patients with an overall sensitivity of 49%, 5%, and 1% to 8%, respectively, in each group.

The implementation of fluorescent primers and laser detection system (F-MSP technique) further increased (up to 55%) the detection rate of *p16* hypermethylation in plasma DNA samples from NSCLC patients.[40] An et al.[41] demonstrated that application of seminested MSP might additionally enhance the sensitivity of *p16* methylation analysis, resulting in 73.3% positivity in lung cancer patients, a value significantly higher than that reported by other studies. Moreover, the recent technologic innovation of microchip electrophoresis offers rapid and accurate analysis of the PCR products from the methylated *p16* gene in plasma DNA, with a positive rate >26.67% higher than that with slab gel electrophoresis, allowing detection in 13 of 48 (27%) lung cancer patients.[42]

Plasma DNA samples from lung cancer patients have also been studied for other biomarkers typically evaluated in tumor tissue, mainly loss of heterozygosity and allele shifts. As early as 1996, Chen et al.[36] reported the presence of MAs (three markers) in 15 of 21 (71%) plasma samples from SCLC patients. Next, Gonzalez et al.[43] screened 35 small-cell lung cancer (SCLC) patients for alterations of three polymorphic markers and demonstrated that 56% of 16 available tumor samples showed molecular changes precisely matching those observed in plasma DNA. Furthermore, Beau-Faller et al.[44] evaluated 12 microsatellite markers targeting nine different chromosomal regions and showed a sensitivity of 85% in detecting lung cancer–associated alterations in 17 of 20 plasma DNA samples from NSCLC and SCLC patients. Importantly, they observed no alterations in plasma or bronchial biopsy DNA from 20 control patients.

However, Sanchez-Cespedes et al.[45] with four markers selected, as well as Sozzi et al.[31] and Bearzatto et al.[40] evaluating five different markers, demonstrated alterations in only 28%, 24%, and 32% of NSCLC cases, respectively. Such a divergence between the increased number of markers and the decreased positive frequency observed might be attributable to the lack of uniformity in the markers and specimen selection, the low number of evaluated patients, and the considerable variety of lung cancer histologic types. Moreover, the sensitivity of microsatellite PCR assays is quite low and may vary significantly depending on the method used for detecting the amplification products. Note also that differences in the allelic alterations in paired plasma and tumor DNA samples might be due to the presence of heterogeneous tumor clones with diverse access to the bloodstream or to the insufficient tumoral microdissection technique, with too many normal cells masking the loss of heterozygosity pattern of tumor cells.

Some studies have also suggested that introducing different marker sets for SCLC and NSCLC would considerably improve their discrimination rate. Nevertheless, several comparable studies that applied uniform marker panels

(ACTBP 2, UT 762, and AR) had analogous characteristics of patient groups (age, sex, and stage), used identical DNA extraction protocols and PCR conditions, and produced discordant results.[36,45] Thus, it should not be forgotten that the high rate of artifacts unique to PCR of minute quantities, for example, the phenomenon of allele dropout, appears to be related not only to the PCR running conditions, but also to the techniques allowing DNA preparation from single cells (incomplete cell lysis) and sample handling (pipetting accuracy and contamination).

Several studies have also evaluated the *TP53* and K-*ras* mutations in serum or plasma, with quite disappointing results. In the study by Gonzalez et al.,[43] only six of 35 SCLC patients exhibited a *TP53* mutation in their tumor and plasma DNA samples. Meanwhile, Andriani et al.[46] identified *p53* genomic mutations in 26 (40.6%) of 64 tumor DNA (NSCLC) and 19 (73.1%) corresponding plasma samples by implementing a plaque hybridization assay and direct DNA sequencing. Ramirez et al.,[38] who analyzed the K-*ras* marker (codon 12) in tumor and paired serum DNA of 50 resected NSCLC patients, detected mutations in nine tumors and 12 serum samples; only one patient carried the same mutation in the tumor and serum. Negative results have also been published by Bearzatto et al.,[40] who found no K-*ras* mutation in the plasma DNA of 35 NSCLC patients, and by Trombino et al.,[47] who observed no difference in the number of K-*ras* mutations in the plasma of NSCLC patients and healthy volunteers. Consequently, the prognostic role of K-*ras* point mutations evaluated in the serum DNA of NSCLC patients needs further confirmation.

Note that a combination of different marker types in plasma DNA, such as gene mutation, microsatellite instability, and DNA quantification, has dramatically increased the percentage of lung cancer patients identified. The diagnostic panel of the *TP53* mutation and three polymorphic markers enhanced the sensitivity up to 71% (at least one molecular change precisely matching that of the primary tumor).[43] Similarly, simultaneous evaluation of *p16* methylation status, MAs, and DNA quantification guaranteed an identification rate of 62% to 80% in NSCLC patients.[40] Accordingly, positive detection of *TP53*, *FHIT*, and *3p* markers effectively identified 68.2% (15 of 22) of stage I patients with similar alterations in the tumor.[47]

The reviewed reports on the evaluation of biomarkers in the plasma clearly demonstrate that none of the analyzed combinations have sufficient sensitivity and specificity for clinical use. Likewise, diagnostic techniques applied in the above-mentioned studies differ significantly. Hence, divergent or even contradictory results were presented in several reports. Thus, standardization of blood sample collection and DNA assay procedures, as well as larger, better matched case-control series are critical for the validation of competent and comparable results.

Apart from the plasma DNA studies, several interesting reports concerning tumor-related circulating mRNA in the peripheral blood of lung cancer patients should also be mentioned. The most promising results have been reported for thyrosinase (5T4) mRNA (in 43% of lung cancer patients and 12% of healthy controls) and for the combination of hnRNP B1 and Her2/neu mRNA markers in serum (all NSCLC and SCLC patients examined).[48,49] Other evaluated mRNA markers either lacked sensitivity (MAGE-2, PGP, and TTF-1) or specificity (CK19). Subsequently, hnRNP B1 mRNA and *hTERT* mRNA markers were assessed and showed a positive detection ratio in 44.5% and 12% of NSCLC patients, respectively.[50,51] These data demonstrate the necessity for further comparative studies on larger groups that will yield more conclusive results and allow better evaluation of the potential diagnostic value of circulating RNA testing.

SUMMARY

Recent developments in the genetic diagnostics of lung cancer promise to provide clinicians with new, efficient noninvasive tools that are useful for screening and early detection of lung cancer. Analysis of genetic alterations in circulating DNA has been particularly noteworthy, as expression of the biomarkers in plasma corresponds adequately with their presence in tumors. Importantly, the amount of sample required is limited and is easily and inexpensively accessible. However, further detailed validation of respective plasma-derived biomarkers is necessary in large multicenter clinical studies, with relevant attention paid to the methodologic issues.

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